larger endoplasmic reticulum and Golgi apparatus than macrophages of nonsmokers (Harris *et al* 1971a,b) indicating that the macrophages from smokers have a higher rate of protein synthesis. Cytoplasmic inclusions have also been found in macrophages from human cigarette smokers (Pratt *et al* 1969; Harris *et al* 1971a; Pratt, Smith, Ladman and Finley 1971; Martin 1973). The inclusions are probably phagolysosomes which can contain needle like structures (Pratt *et al* 1971). The alveolar macrophages from rabbits exposed to cigarette smoke for 20 days have less fenistrae than control macrophages, when examined with the scanning electron microscope (Aranyi 1972).

Cultured alveolar macrophages from cigarette smokers have been reported to exhibit a higher oxidative metabolism than the macrophages from nonsmokers (Harris *et al* 1971a,b) but histochemical studies have shown a decrease in the oxidoreductive enzymes of the alveolar macrophages from smokers (Roque and Pickren 1968). The decrease in enzyme activity was proportional to the amount of fluorescent material, from cigarette smoke, stored by the macrophage. This histochemical study also reported a decrease in acid hydrolase activity (Roque and Pickren 1968) which is at variance with the results of Martin (1973) which described highly elevated activities of acid hydrolase and other hydrolytic enzymes. Macrophages lavaged from the lungs of mice exposed to cigarette smoke have been shown to have a higher rate of RNA metabolism than macrophages from control mice (Holt and Keast 1973b).

Alveolar macrophages from cigarette smokers have an increased ability to adhere to glass (Mann, Cohen, Finley and Ladman 1971). Acute cigarette smoke exposure has been reported to decrease the locomotion and oxidative metabolism of oral leucocytes (Eichel and Shahrik 1969). The alveolar macrophages from cigarette smokers are able to migrate from capillary tubes, faster than the macrophages from nonsmokers (Warr and Martin 1973). The migration of the macrophages from smokers was not inhibited by MIF (Warr and Martin 1973). The inhalation of cigarette smoke has been shown to kill the macrophages in the lungs of mice but some resistance to this acute toxic effect was developed after chronic inhalation of cigarette smoke (Holt and Keast 1973a).

(d) Phagocytosis after exposure to cigarette smoke *in vivo*

No difference has been found between the phagocytic activity of macrophages cultured *in vitro* from human cigarette smokers or non-smokers (Cohen and Cline 1971; Harris *et al* 1971a; Mann *et al* 1971). However guinea pigs exposed to cigarette smoke for four weeks had a depressed pulmonary phagocytic activity (Rylander 1971b) even though cigarette smoke increased the number of macrophages in their lungs (Rylander 1971a). It has also been reported that after two years exposure to cigarette smoke the phagocytic mechanism in the lungs of mice appeared to be overwhelmed by cigarette smoke deposits; large areas of the lung parenchyma were flooded with pigment in the absense of macrophages (United States Public Health Service 1964). Similar overloading of macrophages with particulate matter has been reported (Rylander 1969; Bowden 1971). Lentz and DiLuzio (1973) have reported that the alveolar macrophages of rabbits exposed to cigarette smoke have a normal phagocytic activity but the smoke exposure does decrease the activity of a substance in the lungs which promotes phagocytosis.

The phagocytosis of particles in the lungs can be inhibited by acute exposure to cigarette smoke (Sanders *et al* 1971). The elimination of viable bacteria from the lungs of mice is also inhibited by acute exposure to cigarette smoke (Laurenzi, Guarneri, Carey and Endriga 1963; Laurenzi, Guarneri, Endriga and Carey 1963; Laurenzi, Guarneri and Endriga 1965). This elimination of viable bacteria, which is primarily due to the action of alveolar macrophages (Green and Kass 1964a,b), was not influenced by exposure to cigarette smoke in a strain of mice with a rapid rate of bacterial elimination (Laurenzi *et al* 1963). In order for exposure to cigarette smoke to inhibit the elimination of viable bacteria from the lungs it was found that intestinal bacteria or an intraperitoneal inoculation of endotoxin was required (Laurenzi, Guarneri and Engriga 1965). Chronic and acute exposure of animals to nitrogen dioxide, a component of cigarette smoke, also decreases phagocytic activity and the rate of elimination of viable bacteria from their lungs (Ehrlich, Henry and Fenters 1970; Acton, Myrvik and Winston-Salem 1972; Goldstein, Eagle and Hoeprich 1973).

The phagocytic activity of peripheral blood leucocytes from smokers has been reported to be less than the activity of the

leucocytes from nonsmokers (Kadlubowski (1950) cited by Larson *et al* 1971). The same results were obtained with rabbits after acute and chronic exposures to cigarette smoke (Bruni (1931) cited by Larson *et al* 1971). This was not caused by a decrease in the opsonic activity in the serum. Nicol and Cordingley (1964) have shown that the chronic administration of nicotine to mice does not affect their rate of clearance of particles from blood.

(e) Metabolism of macrophages exposed to cigarette smoke *in vitro*

During experiments in which lung explants from foetal mice were exposed to cigarette smoke for several weeks in culture, Leuchtenberger and Leuchtenberger (1969, 1970b) observed that macrophages were very susceptible to the toxic effects of cigarette smoke. They reported a decrease in both the number of macrophages and the RNA systhesis of the macrophages, as judged by histochemical techniques. Monolayer cultures of alveolar and peritoneal macrophages have been found to be more susceptible to the toxicity of cigarette smoke than either fibroblastic or epithelioid cells from lungs of foetal mice (Holt and Keast 1973b; Holt, Bartholomaeus and Keast 1974). Macrophages which survived the exposure to cigarette smoke had a higher rate of RNA synthesis than control cultures 30 minutes after the exposure, but their RNA synthesis was decreased 24 hours after the exposure. Macrophages with a high basal rate of RNA synthesis were less susceptible to the toxic effects of the cigarette smoke (Holt and Keast 1973b). Yeager (1969) and Holt and Keast (1973d) have shown that the protein synthesis of macrophages is decreased by the acute exposure to solutions of cigarette smoke but macrophages repeatedly exposed to low levels of smoke have a marked increase in RNA and protein synthesis (Holt and Keast 1973d). Cigarette-smoke solutions have been shown to decrease the oxygen consumption of macrophages (York *et al* 1973) and Lentz and DiLuzio (1972, 1973) have reported that cigarette smoke depresses the oxidation of glucose by macrophages. Other experiments have shown that the activity of enzymes isolated from alveolar macrophages can be inhibited by solutions of cigarette smoke (Powell and Green 1971). Nicotine has also been shown to decrease the oxygen consumption and adenosine triphosphatase activity of alveolar macrophages (Meyer, Cross, Ibram and Mustafa 1971).

Thus in general the results of the experiments *in vitro*

show that cigarette smoke has an acute toxic effect on macrophages but prolonged cigarette smoke exposure can produce macrophages with an elevated metabolic activity. This concurs with the observations of Harris *et al* (1971a,b) who examined the alveolar macrophages from cigarette smokers.

(f) Phagocytosis after exposure to cigarette smoke *in vitro*

Green and Carolin (1967) have shown that cigarette smoke could inhibit the killing of *Staphylococcus albus* by rabbit alveolar macrophages. The active component was in the vapour phase of the cigarette smoke, but nicotine, acetaldehyde, formaldehyde and cyanide, in doses comparable to their concentration in cigarette smoke did not affect the macrophages. Subsequently it was found that glutathione or cysteine could protect the macrophages (Green 1968). If the inactivation of bacteria was measured in the absence of serum there was a 30% reduction in killing activity, but the bacteriocidal activity was either not affected or slightly increased by cigarette smoke. In the presence of serum, however almost 100% of the phagocytic activity of the macrophages could be decreased by an exposure to cigarette smoke. Lentz and DiLuzio (1972, 1973) reported that the killing and degradative mechanisms of alveolar macrophages were not impaired by cigarette smoke but the ingestion of bacteria and other material was inhibited. They have also reported that cigarette smoke did not impair phagocytosis by liver macrophages. The phagocytic activity of alveolar macrophages can be inhibited by the exposure to nitrogen dioxide *in vitro* (Vassallo *et al* 1973).

(g) Humoral immunity

Experiments with animals have shown that the inhalation of cigarette smoke can decrease serum antibody titres. Donzelli (1933) (cited by Silvette *et al* 1957) found that inhalation of cigarette smoke decreased antibody titres of rabbits immunized with typhoid. From a recent study, Esber, Menninger, Bogden and Mason (1973) reported that both primary and secondary antibody responses of mice to sheep erythrocytes were inhibited by short term cigarette-smoke exposure. The inhibition of antibody response could be detected two weeks after exposing the mice to cigarette smoke for one week.

A survey of serum antibody titres, to influenza virus, of military cadets has shown that cigarette smokers initially responded to infection or vaccination as well as nonsmokers (Finklea, Hasselblad, Riggan, Nelson, Hammer and Newill 1971). However the persistance of hemagglutinating antibody to influenza antigen was decreased in cigarette smokers when compared to nonsmokers. It has also been reported that smokers have a depressed level of natural agglutinins of sheep erythrocytes (Fletcher, Sumney, Langkamp and Platt 1968), but the ages and health of the smokers were different from those of the nonsmokers examined. Another investigation, however, found cigarette smokers had a higher titre of antibody (precipitin) to *Haemophilus influenzae* than nonsmokers (May, Peto, Tinker and Fletcher 1973). It was concluded from the results of a multiple regression analysis that the stimulation of these serum antibody levels was not dependent on respiratory infection. This analysis did not consider subclinical infections but in any case stimulation or inhibition of serum antibody responses to respiratory infection by cigarette smoking may only reflect an abnormality of defense mechanisms in the respiratory tract. Human cigarette smokers have also been found to have increased serum C-reactive protein levels and an abnormal seroflocculant for ethyl choledienate (Heiskell, Miller, Aldrich and Carpenter 1962).

Experiments performed *in vitro* have shown that nicotine or aqueous extracts from cigarette smoke rapidly and irreversibly inhibited an early event in the induction of antibody from immunocompetent cells by antigen (Roszman and Rogers 1973). The concentration of smoke solution used in these experiments did not influence the viability of the lymphocytes.

Components of cigarette smoke have been shown to both inhibit and stimulate antibody responses. Guardasciope (1942) (cited by Silvette *et al* 1957) found that nicotine could decrease antibody responses of rabbits to ox erythrocytes. Chronic exposure to nitrogen dioxide increased the serum neutralising antibody titres to influenza infection in squirrel monkeys (Fenters, Findlay, Port, Ehrlich and Coffin 1973). Inhalation of carbon particles caused a transient stimulation of the antibody-forming cell response in mediastinal lymph nodes of mice to an aerosol of *Escherichia coli* (Zarkower 1972a,b). The response in these nodes was eventually decreased as well

as in the spleen where no transient enhancement occurred.

(h) Cell-mediated immunity

There is little information relevant to cell-mediated immunity in cigarette smokers. The vapour phase of cigarette smoke has been shown to decrease the number of human peripheral blood lymphocytes which transform in the presence of phytohemagglutinin (PHA) (Desplaces, Charneine and Izard 1971). This suggests the possibility that the activation of thymus-dependent lymphocytes, by antigens, may be impaired by cigarette smoke (Rodey and Good 1969; Adler *et al* 1970).

The recent report that alveolar macrophages of cigarette smokers will not respond to MIF indicates that an important defect of cell-mediated immunity may exist in the lungs of cigarette smokers (Warr and Martin 1973).

(i) Tracheobronchial clearance

Although the mucociliary clearance mechanism of the bronchi and trachea is not strictly a part of the immune system it constitutes an important pulmonary defense mechanism (Green 1970; Gross and Detreville 1972). Many reports have shown that the ciliary action of the respiratory epithelium of several animals can be inhibited by cigarette smoke (United States Public Health Service 1964). However the question of whether the clearance of inhaled particles from the lungs of cigarette smokers is depressed is unresolved. Rylander (1971) has shown that the chronic exposure of guinea pigs to cigarette smoke depresses the tracheobronchial clearance of inhaled bacteria. The clearance of inhaled particles from donkeys chronically exposed to cigarette smoke is also depressed (Albert, Spiegelman, Shatsky and Lippmann 1969) but La Belle, Bevilacqua and Brieger (1966) did not find an impairment of tracheobronchial clearance in rabbits exposed to cigarette smoke over a period of fifteen days. Experiments measuring the clearance of radiolabelled particles inhaled by humans have been conducted. Cigarette smoking has been shown to stimulate the tracheobronchial clearance of cigarette smokers (Camner, Philipson and Arvidsson 1971). Albert, Lippmann and Briscoe (1969) reported that some cigarette smokers had impaired clearance mechanisms but Thomson and Pavia (1973), who conducted a larger study, did not find an impairment in cigarette smokers. It has been reported that the

tracheobronchial clearance of smokers improves after discontinuing smoking (Camner, Philipson and Arvidsson 1973). Camner and Philipson (1972) also found that in five out of ten monozygotic twins with discordant smoking habits, the smoking twin had a slower clearance of inhaled particles. The evidence indicating that tracheobronchial clearance is impaired in cigarette smokers is supported by morphological observations showing alterations and replacement of ciliated cells in the respiratory epithelium of cigarette smokers (Chang 1957; Auerbach *et al* 1962). It is probable that the impairment of the clearance mechanisms in an isolated area of the tracheobronchial system can have a considerable influence on health.

E. Concluding remarks

It is apparent from the review of the literature that information concerning immunity in cigarette smokers is fragmentary and often controversial but there are suggestions that humoral and cell-mediated immunity may be impaired in cigarette smokers. Research into the effect of cigarette smoking on the immune system appears to have been mainly directed towards studying the effect of cigarette smoke on macrophages. However the bacteriocidal and phagocytic capacity of the macrophages in the lungs of human cigarette smokers is not known.

While the diseases associated with cigarette smoking can be considerably reduced if people discontinued smoking there are indications that a substantial proportion of cigarette smokers will continue to smoke and that a large number of people will adopt the habit (Workship of the Second World Conference on Smoking and Health 1972). It has been recognised that as well as discouraging cigarette smoking there is a need to develop cigarettes which are less injurious to health and methods of preventing the diseases occurring in cigarette smokers. The latter possibilities require the identification of the harmful component(s) of cigarette smoke and their mechanism of action.

The experimental studies in the following chapters describe investigations into the immunological competence of mice chronically exposed to cigarette smoke. Humoral and cell-mediated immunity and phagocytic function have been examined. Some studies on the action

of cigarette smoke on components of the immune system *in vitro* have been described. The relevance of these investigations to the concept that immunosuppression, produced by the inhalation of cigarette smoke, may be involved in the aetiology of the disease patterns found in human cigarette smokers and in animals exposed to cigarette smoke, has been discussed.

# CHAPTER 2

## MATERIALS AND METHODS

A. Exposure to cigarette smoke

(a) Exposure of mice

Mice were exposed to cigarette smoke in a Hamburg II inhalation apparatus (Heinr Borgwaldt; West Germany). The apparatus (Fig. 1) has been developed and described by Dontenwill (1970). The noses and mouths of the mice were admitted to the inhalation chamber which was supplied with a fresh mixture of smoke diluted in air at a ratio of 1:7 (smoke:air) every other second. The cigarette smoke was supplied by a 35ml "puff" from an individual cigarette. The mice were exposed to 30 cigarettes per day in cycles in which a puff was taken from each cigarette (7 minutes continuous cigarette smoke exposure). King sized filter cigarettes supplied by the Australian Tobacco Research Foundation were used for all studies. Except where otherwise specified mice were 6 weeks old at the commencement of their exposure period.

Because of the facilities available, control mice were not placed in the smoking apparatus without cigarettes. Other studies have shown that placing mice in an inhalation apparatus for prolonged periods did not influence body-weight (Leuchtenberger and Leuchtenberger 1970a) and similar handling of animals, 3 times a day, over a period of 2 weeks did not depress the immune response (Esber *et al* 1973). Judging by the movements of the mice it was observed that the actual exposure to smoke produced the most stress and that mice became accustomed to the smoke after several weeks of exposure. Accordingly experiments were designed to differentiate between the effects of chronic exposure and the effect of short-term exposure, where stress reactions were most expected.

(b) *In vitro* exposure to cigarette smoke

Cell cultures were exposed to cigarette smoke in a perspex chamber (Fig. 2), designed to produce fresh cigarette smoke-air mixtures from one cigarette in a ratio of 1:7 (smoke:air). The chamber was designed and reported by Holt and Keast (1973b). The exposure cylinder (F) was 27cm long and 5.8cm in diameter. In order to disperse the cigarette smoke a wire coil was placed in the outlet from the cigarette holder (D). The tissue cultures in 35mm petri dishes were placed in position (E), which was 15cm from the

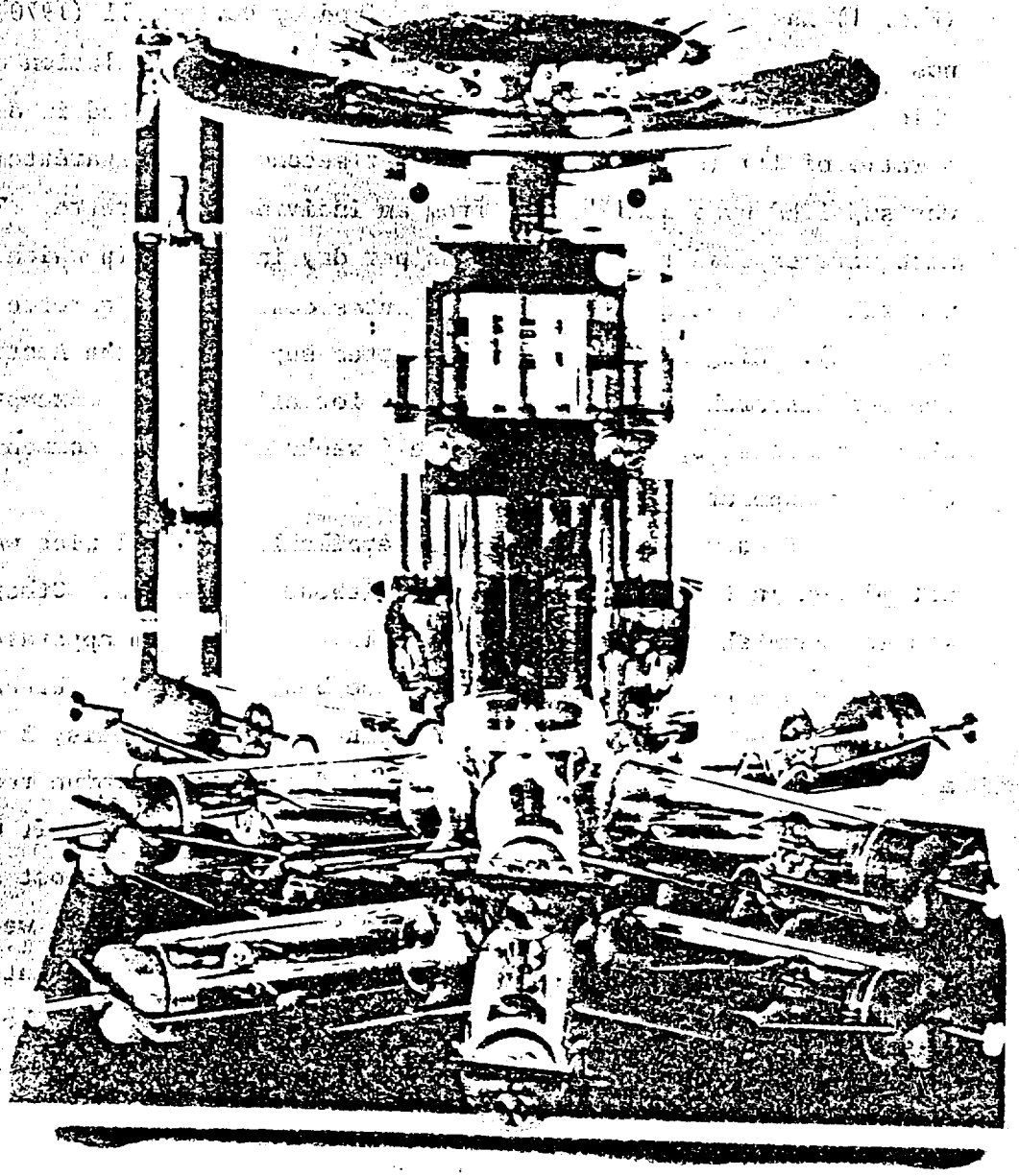

FIGURE 1 Hamburg II smoking machine.

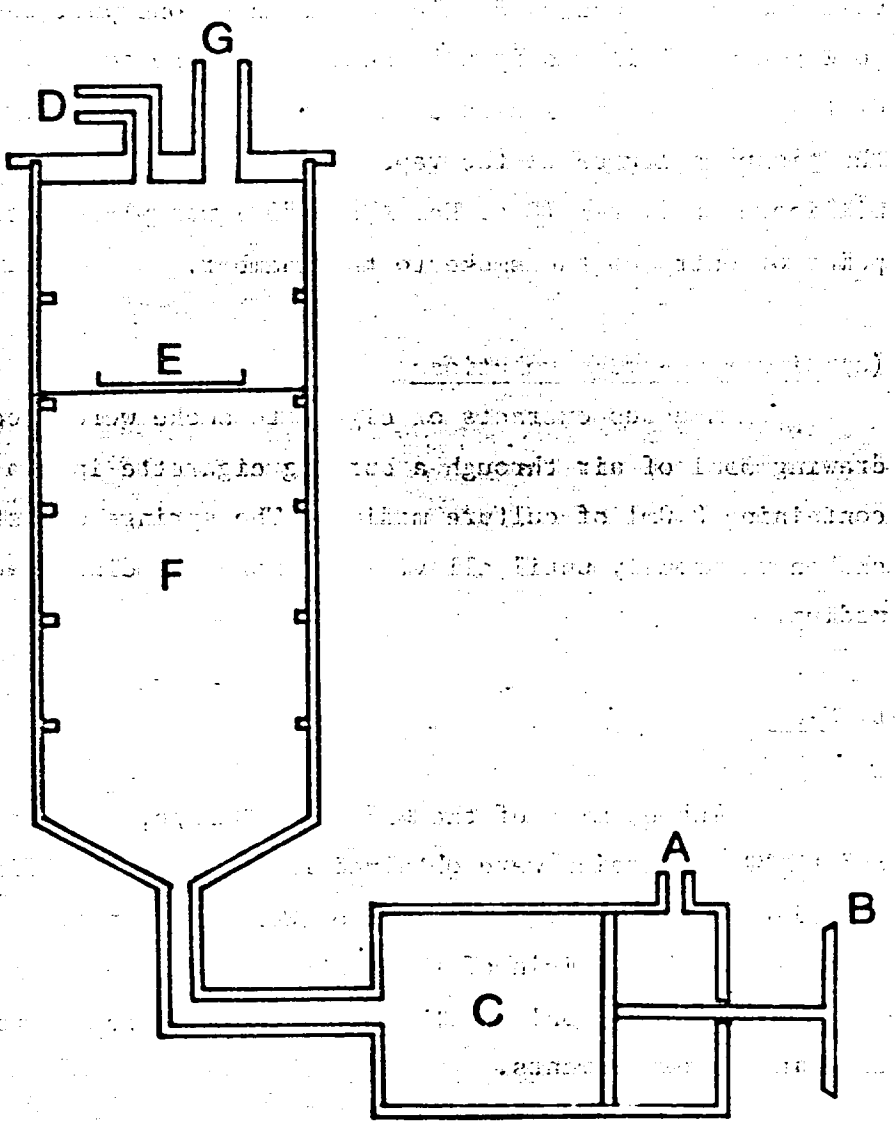


FIGURE 2 Tissue culture smoking machine: vacuum line (A); plunger (B); syringe (volume at position shown (100ml) is 1/7 volume of exposure chamber) (C); cigarette holder (D); tissue culture dish (35mm) 8cm from smoke inlet (E); perspex smoke chamber (30cm in length) (F); outlet to atmosphere (G). To introduce a puff of smoke, outlet G is blocked and plunger (B) is fully withdrawn to position shown. After 2 seconds, the cigarette is removed from D and the plunger (B) is fully withdrawn. The smoke is rapidly cleared from the smoke chamber (F) via the vacuum line (A). After 58 seconds the tissue culture dish is removed from the chamber or when required the cycle repeated.

entry of the cigarette smoke. In order to produce the smoke a puff volume of 100ml was used. The duration of one puff was standardised to 2 seconds followed by a 58 second exposure to air. A description of the operation is included in the legend of Fig. 2. To expose the tissue cultures to the vapour phase a millipore filter (45μ) and millipore prefilter (Cat. No. AP2004700) was placed across the point of entry of the smoke to the chamber.

(c) Cigarette-smoke solutions

Aqueous extracts of cigarette smoke were produced by drawing 35ml of air through a burning cigarette into a 50ml syringe containing 8.0ml of culture medium. The syringe was stoppered and shaken vigorously until all visible smoke was dispersed in the medium.

B. Mice

Inbred mice of the C57Black, BALB/c, Simpson, C3H, DK Ginger and DK Black strains were obtained from the Perth Medical Centre animal breeding unit (University of Western Australia, Perth, Western Australia). The origin of these strains have been described by Cox and Keast (1973). Unless otherwise specified female mice were used in all experiments.

C. Body weights, organ weights and food consumption

The moist weight of the food and organs and the body weight were determined with the use of a top loading balance (Mettler Model P160, Greifensee - Zurich, Switzerland).

D. Carboxyhaemoglobin (HbCO) estimation

The method for determining the HbCO was adapted from Salt (1951). The percentage haemoglobin not reducable by sodium dithionate was used as a measure of the % HbCO. 50μl of blood were withdrawn from the retro-orbital venous plexus of mice and added to 6ml of 0.1% ammonia solution. The optical density at 600nm was determined by a spectrophotometer (SP 800, Unicam Instruments, Cambridge, England), ($R_1$). 0.2ml of freshly prepared 10% sodium dithionate was added and.

the optical density at 600nm measured ($R_2$). The value $R_2 - R_1$ was calculated for each mouse. As inbred mice, with little variation in haemoglobin concentration, (See tables 5 - 8), were used the %HbCO was calculated:

$$\%HbCO = 100 - \frac{R_2 - R_1 \text{ (test)}}{R_2 - R_1 \text{ (control)}}$$

Groups of 5 test and age-matched controls were used for each determination.

E. Haematology

Blood was collected from the retro-orbital venous plexus of mice in 20µl capillary tubes, (Microcaps, Drummond Scientific Co., Bromall, Philadelphia, U.S.A.), suspended in 10ml of isoton (Nicol and Davis 1971) and haematological data was determined using a Coulter Counter (Model S) (Coulter Electronics Inc., Hialeah, Florida U.S.A.) operated by Mrs. L. Zosky, Division of Haematology, Repatriation Hospital, Perth, Australia). Differential leucocyte counts were performed with May-Grünwald-Geimsa stained blood smears by methods described by Dacie and Lewis (1968).

F. Humoral immune responses to sheep erythrocytes

Sheep erythrocytes (SRBC) were collected in Alsever's solution and stored at $4^{\circ}C$ for periods of up to one week. All SRBC preparations were washed x 3 in phosphate buffered saline (PBS) before use.

(a) Inoculations

Mice were inoculated intravenously into the tail vein or intraperitoneally with $10^8$ SRBC in 0.1ml of PBS. For intratracheal inoculations (Thomas, Holt and Keast 1974a) mice were anaesthetised with pentobarbitone sodium (Nembutal, Abbott Laboratories, Australia), and a blunt 19 gauge needle inserted under the epiglottis and almost to the base of the trachea where $10^8$ SRBC in 0.01ml of PBS were introduced using an Alga micrometer syringe (Burroughs Wellcome and Co., London, England).

(b) Plaque-forming cell (pfc) determination

(i) Cell preparations

Mice were exsanguinated and the blood cells allowed to sediment in a final concentration of 0.6% dextran 110 (Glaxo Intradex NCIB 8710) containing 7IU heparin/ml. After one hour the buffy coat was collected and washed twice in growth medium (Eagle's minimal essential, Grand Island Biological Co., F15). The cells were then suspended in 0.2ml of medium. The mediastinal and cervical lymph nodes were removed and pooled, except where indicated. The nomenclature concerning the lymph nodes has been adapted from Dunn (1954). The lungs, spleen and lymph nodes were washed in PBS, then finely minced and suspended in medium. The suspensions were filtered through nylon wool and then washed. The spleen cells were suspended in 2.0ml, and the lymph node and lung in 0.2ml of growth medium. The concentrations of leucocytes obtained from spleen and lymph nodes were about $10^8$ and $10^7$ cells/ml respectively. Lymphocytes were the predominant type of cell in both preparations. About $5x10^6$ cells/ml could be obtained in the preparations of lung cells. These were 80% - 90% lymphocytes with the remainder consisting of macrophages. Few epithelioid cells were present.

(ii) Haemolytic-plaque assay

The haemolytic-plaque assays were performed in duplicate. 0.02ml aliquots of cell suspension, guinea pig complement, 8%SRBC suspension in PBS and either PBS or a suitable concentration of rabbit anti-mouse globulins were mixed and placed in the chambers similar to those described by Cunningham and Szenburg, (1968). The anti-mouse globulins antiserum was a gift from K. C. Cox (Department of Microbiology, University of Western Australia). The chambers were incubated at 37°C for 30 minutes before counting the plaques. The indirect pfc values were obtained by subtracting the number of plaques developing without the anti-mouse globulins from the plaques developing with this reagent.

(c) Antibody titres

Antibody assays were performed in microtiter plates (Microtiter trays 220-25A, Cooke Engineering Company, Alexandria Virginia, U.S.A.). Haemagglutinating antibody was determined by serially diluting 0.02ml of serum in an equal volume of PBS containing 1% normal rabbit serum. For the reaction 0.02ml of 0.25% SRBC

in PBS was added to the dilutions. Haemolytic antibody was measured in the same manner using PBS as a diluent and adding 0.02ml of guinea pig serum as a source of complement activity, and 0.02ml of 1% SRBC in PBS to each dilution. The highest dilutions showing complete lysis or definite agglutination were read after 1 hour at 37°C.

G. Antibody response to Polyvinylpyrrolidone (PVP)

Mice were inoculated with 0.2µg of PVP-360 (MW 360,000) (Sigma, St. Louis, Missouri U.S.A.), in 0.1ml of PBS into the tail vein. After one week the serum antibody titre was measured by indirect haemolysis of PVP coated SRBC as described by Andersson and Blomgren (1971) and Andersson (1969). To couple the SRBC and PVP, the SRBC were washed x 3 in PBS and resuspended to 10%. To 10ml of the 10% SRBC, 0.05ml of a 0.01g/ml solution of tannic acid in PBS was added and the mixture incubated for 10 minutes at ambient temperature. The SRBC were then washed x 2 in PBS and resuspended in 10ml and then 0.1ml of 0.01g/ml of PVP (MW 24,500) was added. This mixture was incubated for 10 minutes at ambient temperature before washing x 3 in PBS and resuspending in PBS.

The indirect haemolytic antibody titre was determined by the method described for the haemolytic antibody titre to SRBC except that 2% tanned SRBC were used. Preliminary tests showed that normal mouse serum did not lyse the PVP coated tanned SRBC and that a pool of serum from 10 mice immunized with PVP did not lyse the untreated tanned SRBC. The reaction required complement and could be completely inhibited by adding PVP (MW 24,500) at a final concentration of 2.5mg/ml.

H. Preparation of rabbit anti-SRBC antiserum fractions

Two rabbits were immunized intravenously with $10^9$ SRBC and then intraperitoneally with $2x10^7$ SRBC after 12 days. Serum was collected from the animals 12 and 27 days after the first inoculations. All sera were pooled and fractionated by Sephadex G-200 chromotography using PBS as an eluent. 3ml of serum was fractionated in a column (2.5cm x 100cm), by the upward flow method at a flow rate

of 15ml/hour. 6ml fractions were collected and monitored for absorption at 280nm in a spectrophotometer. Three well defined peaks were obtained. The eluates were pooled into 2 fractions as shown (Fig. 3) and concentrated by ultrafiltration using a Diaflow PM-10 membrane (Diaflow Ultrafiltration membranes, Amicon, Lexington, Massachusetts, U.S.A.). Fraction I and fraction II (Fig. 3) were designated 19S and 7S fractions respectively as other studies have shown that these fractions would contain 19S or 7S antibody (Thomas, Turner, Eadie and Yadav 1972).

I. Phytohaemagglutinin (PHA) stimulation

The micromethod used to determine the incorporation of $^{3}$H-6-thymidine (Radiochemical Centre, Amersham, U.K.) into lymphocytes in the presence of PHA-P (Difco Laboratories, Detroit, U.S.A.) has been described in detail by Keast and Bartholomaeus (1972). Mice were killed by cervical dislocation and 20IU of sodium heparin inoculated into the thorax and the heart severed. Blood was collected from the thoracic cavity. The spleens and lymph nodes of mice were finely minced using aseptic techniques, and suspended in 3ml of growth medium (RPMI 1640 (Grand Island Biological Co., New York, U.S.A.)) supplemented with 10% heat-treated calf serum (60$^{o}$C for 2 hours). After allowing large tissue fragments to sediment by gravity the cell suspensions were purified by centrifugation on a mixture of Ficoll (Pharmacia, Uppsala, Sweden), and Hypaque (Winthrop Laboratories, New York, U.S.A) at 500g for 25 minutes (1ml of 34% (w/v) Hypaque to 2.4ml of 9% (w/v) Ficoll). The blood was made up to 3ml with growth medium and purified by centrifugation on a mixture of Ficoll and Hypaque. The leucocyte preparations were washed x 2 in growth medium (using centrifugation at 200g for 10 minutes) and resuspended in the medium. $5x10^{4}$ lymphocytes were then cultured in 0.2ml of medium containing either 0, 0.25, 1.25, 2.5, 4, 10, 20 or 50µg of PHA, in wells of Microtest II trays (Falcon Plastics Cat. No. 3040 and 3041). Triplicate cultures were set up for each concentration of PHA. After 24 hours incubation 0.5µCi of $^{3}$H-6-thymidine (5Ci/mmole) was added to each culture and incubation continued for a further 24 hours. The plates were then centrifuged at 600g for 10 minutes using a centrifuge fitted with an attachment to hold Microtest plates (Cooke Engineering,

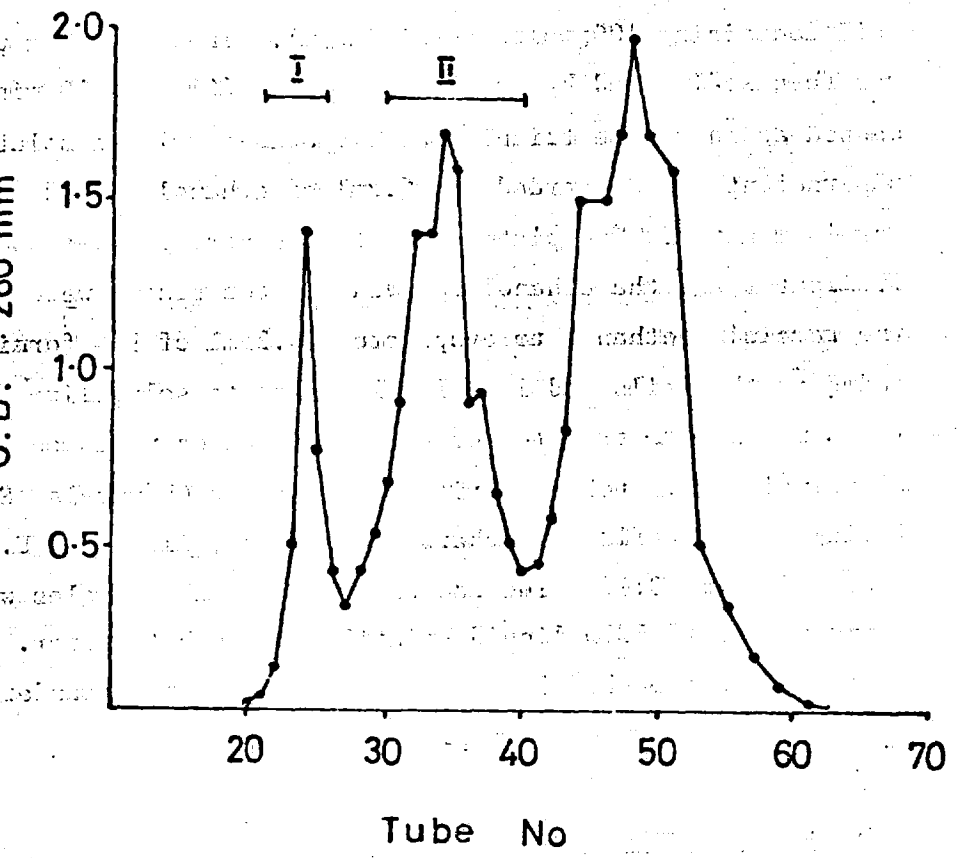


FIGURE 3 Fractionation of 3ml of rabbit serum in a Sephadex-G200 column (2.5 x 100cm). Tube volume 6.0ml.

Alexandra, Virginia U.S.A.). The supernatant was removed and the cells washed x 2 in PBS containing 100μmoles thymidine/ml. 5μl of a 25mg/ml solution of bovine serum albumin were then added to the cell pellet in each well followed by 0.2ml of 5% trichloroacetic acid containing 100μmoles thymidine/ml. The resulting precipitate was then sedimented by centrifugation at 600g for 10 minutes and washed again in the trichloroacetic acid-thymidine solution. The supernatant was discarded and 0.2ml of ethanol was added to wells for 10 minutes. The plates were then centrifuged at 600g for 10 minutes and the ethanol removed and the plates were left for the remaining ethanol to evaporate. 0.25ml of 90% formic acid were added to the wells and left for 24 hours to solubilize the samples. The samples were transferred to scintillation vials containing 10ml of scintillant: toluene:triton-X 100 (Ajax Chemicals, Sydney, Australia) and Permafluor (Packard Instruments, Illinois, U.S.A.) in a ratio of 332:150:18. The radioactivity in the samples was determined using a Packard 3375 liquid scintillation spectrometer. Counting efficiency approached 50% and automatic external standardisation was carried out on each sample.

J. Pulmonary-bacteriocidal activity

A leucine-requiring strain of *Pseudomonas aeruginosa* was cultured overnight in a nutrient broth containing 50μCi/ml of $H^3$-5-uridine (Radiochemical Centre Amersham, Bucks U.K., 5Ci/mmole), then washed three times in PBS and resuspended in PBS at a concentration of $2x10^9$ bacteria/ml. The number of bacteria was measured with a Helber chamber. Mice were anaesthetised with pentobarbitone sodium and a blunt 19 gauge needle inserted under the epiglottis and almost to the base of the trachea, where 0.01ml of the bacterial suspension was introduced. After a specified time interval, the lungs were then removed from just above the junction of the trachea and bronchi, and homogenised in 0.5ml of PBS. To measure radioactivity, 0.05ml of the homogenate was incubated at 37°C with 0.05ml of hydrogen peroxide for 2 hours, then 1.0ml of soluene (Packard Instruments, Illinois, U.S.A.) was added and the mixture incubated for a further 22 hours. The sample was then transferred to a scintillation vial containing 10ml of a 1:24 mixture

of Permafluor (Packard Instruments) and toluene. Radioactivity was determined in a Packard 3375 liquid scintillation spectrometer. External standardisation was carried out on each sample and counting efficiency approached 30%. The viable-bacterial counts were performed by serially diluting the lung homogenates in PBS and plating 0.1ml of an appropriate dilution on a minimal media plate (containing 1.5% agar in a Vogel and Bonner salt solution (Vogel and Bonner 1956)) with 0.5% glucose, 0.01% leucine and 0.03% cetrimide (cetrimonium bromide) and counting the colonies growing after an overnight incubation. When the dilutions were plated on a plate without leucine, the number of colonies was negligible, indicating that almost all the bacteria normally counted were the leucine-requiring strain originally introduced to the lungs. The counts of viable bacteria and radioactivity were performed in duplicate, and the values compared to a group processed immediately after the administration of bacteria.

K. Systemic reticuloendothelial function

$2 \times 10^8$ bacteria from a preparation of *Pseudomonas aeruginosa* which had been labelled by incubation with $H^3$-5-uridine, were inoculated into the tail vein of mice and immediately ($t_0$) 20µl of blood was sampled from the retro-orbital plexus. After 10 minutes ($t_{10}$) another sample was taken. The phagocytic index was calculated by measuring the disappearance of radioactivity from the blood.

$$K_p = \frac{\log DPM_0 - \log DPM_{10}}{t_{10} - t_0}$$

(t = minutes, DPM = disintegrations per minute).

At 10 minutes and 180 minutes after the inoculation, the radioactivity in the spleen, kidney, liver and lungs was determined. The radioactivity in the organs as well as the blood was counted as described above for the lungs (Section I). The change of the radioactivity in the organs between 10 and 180 minutes was analysed by calculating the slope and standard deviation of the slope of the regression line of radioactivity on time. The significance of the differences in the slopes between control and smoke-exposed animals was calculated by Student's t test (Campbell 1967).

L. *In vitro* phagocytosis and elimination of radioactivity

The method used to measure phagocytosis *in vitro* has been described in detail (Thomas, Holt and Keast 1974b). Peritoneal macrophages were obtained from untreated C57 Black mice and cultured in MEM supplemented with 10% heat treated calf serum (2 hours, 60°C), and cultured on stainless steel squares in 35mm petri dishes containing 2ml of medium as described by Keast and Birnie, (1969) for 20 - 22 hours. Batches of 5 - 15 mice were used for each experiment. Macrophages were plated in petri dishes without stainless steel squares and by counting the number of viable macrophages, as judged by trypan blue exclusion, in 20 fields of 3 dishes, the number of macrophages per stainless steel square was determined (Keast and Birnie 1969). To measure phagocytosis, heat-treated *Pseudomonas aeruginosa* were added to the cultures after changing the medium. Except where indicated $18.8 \times 10^7$ bacteria/ml were used. After the required period for phagocytosis, extracellular bacteria were washed off by directing a stream of PBS (1ml) twelve times across the stainless steel square. The cultures were further rinsed in PBS, fixed in 5% trichloroacetic acid for 10 minutes and then in 3:1 (v/v) ethanol/acetone for 3 minutes before rinsing in acetone. To measure elimination of phagocytosed radioactivity, the macrophages were washed as above and then reincubated in fresh warm medium. After the period of elimination the macrophages were rinsed in PBS and then fixed and dehydrated as above. The radioactivity was measured with a Nuclear Chicago gas-flow detector (Model D47). The specific activity of the bacterial preparations were determined and the results expressed as bacterial equivalents/cell. No cell death occurred when the macrophages were incubated with the bacteria for 5 hours but 60% macrophage death occurred if the incubation with bacteria was continued for 24 hours. However, when macrophages were incubated with the bacteria for 3 hours, then washed to remove extracellular bacteria and reincubated for 24 hours in fresh medium no cell death occurred.

In these studies radiolabelled *Pseudomonas aeruginosa* were prepared by incubating the bacteria in a minimal salts solution (Vogel and Bonner 1956) with 0.5% glucose, 0.001% leucine and 20μCi

of $H^3$-5-uridine. The bacteria were heat-treated for 2 hours at 60°C, washed three times in PBS and stored at 4°C.

To study the effect of exposure to cigarette smoke on the phagocytic capacity of macrophages, their phagocytosis was measured 30 minutes after the exposure unless otherwise specified. The adherence of the macrophages was measured by counting the number of viable macrophages before and after the washing process used to remove bacteria. The adherence was calculated and expressed as the percentage of macrophages remaining on the monolayers after the washing.

M. Growth of tumours

The Lewis lung tumour (Sugiura and Stock 1955) of C57Black mice was a gift from Dr. Hellman of the Imperial Cancer Research Fund, Lincolns Inn Fields, London, U.K., and was maintained by serial transplantation in adult mice. This tumour originated as a spontaneous anaplastic lung carcinoma. A preliminary study showed that the subcutaneous inoculation of $10^6$ viable cells produced tumours in 18/20 mice. This number of viable cells was used as a standard inoculum. The diameter of the tumours was determined by two measurements taken at right angles to each other, employing calipers. The cube of the mean of the diameters determined by this method and the weight of the tumours were found to have a product-moment correlation coefficient of 0.89. The ability of this tumour to metastasise to the lungs was measured by the method of Wexler (1966). The lungs were infused with dilute India ink (15ml of ink in 85ml of distilled water) through the trachea, washed in tap water and then fixed and bleached in Feketes solution (70% ethanol, formaldehyde, acetic acid mixed in the ratio of 100:10:5). In order to allow the tumours maximum opportunity to metastasise, the tumour bearing animals were left until a number died or became moribund (about 30 days).

The TKL5 cells were a clone from a line (W47-A) of tumour cells cultivated *in vitro* from a tumour induced in a newborn BALB/c mouse by murine sarcoma virus (Harvey) (MSV-H) (Thomas, Aw, Papadimitriou and Simons 1973). They were maintained in tissue culture using

McCoys 5A medium (Grand Island Biological Company, New York, U.S.A.), supplemented with 10% heat-treated calf serum (2 hours, 60°C), and produced MSV-H as judged by the ability of tissue-culture-fluid filtrates to produce tumours in newborn mice. These cells were inoculated intratracheally (Section F(a)). The standard inoculum was $10^5$ viable cells per mouse.

N. Histopathology

Histological examination was performed by the techniques of Walters, Leak, Joske and Stanley (1963).